STUDIES ON ERGOTHIONEINE

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STUDIES ON ERGOTHIONEINE

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Contents

page

1. Introduction..... 1
   a) Historical..... 1
   b) Scope of Thesis..... 5
2. The determination of the ergothioneine content of ergot from various plant hosts..... 7
   a) Experimental..... 7
      1) Preparation of test solution..... 7
      2) Colorimetric determination..... 8
   b) Results..... 9
   c) Discussion..... 11
3. The determination of blood ergothioneine levels of various species in normal and abnormal conditions..... 13
   a) Experimental..... 13
      1) The preparation of test solution..... 13
         a. Reagents..... 13
         b. Deproteinization of blood..... 14
         c. Lead acetate precipitation..... 15
         d. Method..... 15
      2) Colorimetric determination of ergothioneine..... 16
         a. Reagents..... 16
         b. Method..... 17
   b) Results..... 21
   c) Discussion..... 22

page

4a. The effect of alloxan diabetes on the ergothioneine level in the blood of rats..... 24

a) Experimental..... 24

b) Results..... 25

c) Discussion..... 27

4b. The effect of intravenously injected ergothioneine on alloxan-induced diabetes in rats... 29

a) Introduction..... 29

b) Experimental..... 31

c) Results..... 32

d) Discussion..... 33

5. Summary..... 35

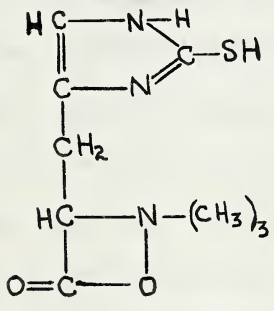
6. Bibliography..... 36

1. Introduction

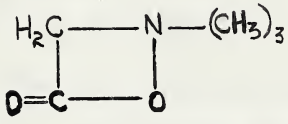
a) Historical

Ergothioneine was isolated and named by the French worker Tanret in 1909 (24). The compound was isolated as the hydrochloride from the ergot of rye by means of water-extraction and mercuric salt precipitation. Because of its source and the presence of sulfur in the molecule, Tanret named the substance ergothioneine. Barger and Ewins in 1911 (2), showed the molecular structure to be the trimethyl betaine of thiolhistidine.

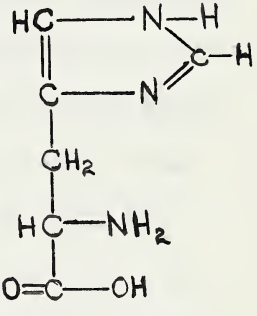
Ergothioneine



Betaine



Histidine



Ergothioneine remained as an obscure, organic base, obtainable from ergot, until 1926 when Benedict, Newton, and Behre at Cornell (7), and Hunter, Bulmer, and Eagles at Toronto (8), independently isolated a sulfur containing base from hog's blood. The methods of isolation used by these two groups of workers differed with the result that the analyses of their

respective products differed. The Americans named their substance thiosine, the Canadians, sympectothion. However Eagles and Johnson (10) showed both substances to be identical with the ergothioneine of Tanret.

In 1928 Hunter devised a Koessler and Hanke type diazo-test for the colorimetric estimation of ergothioneine in simple solutions. Determinations of blood ergothioneine were attempted and values for various species published (12). These values were considered as tentative however.

At the same time Benedict and his co-workers also devised a method for the estimation of blood ergothioneine. After deproteinization with tungstic acid; uric acid, ergothioneine, and glutathione were precipitated as the silver salts by means of silver lactate. The uric acid was selectively dissolved by 10% NaCl in 0.1 N HCl solution. Then the ergothioneine (and perhaps the glutathione) was dissolved by cyanide. The ergothioneine was then determined by means of a uric acid test.

The results of these determinations were not published because of uncertainty of the authors about their method. They found that for some unknown reason, some preparations of sodium tungstate caused removal of ergothioneine during protein precipitation. Some

overall figures for blood ergothioneine in various species were suggested however. They found that pig bloods showed a range of 25 - 30 mg.%, normal humans 14 - 15 mg.%, and diabetic humans a higher range of 20 - 27 mg.% ergothioneine. These results are high when compared to our present findings.

Benedict and his co-workers gradually modified their method. In a later paper (4), using molybdic acid as the deproteinizing agent and sodium hydroxide to dissolve the silver salt of ergothioneine, they give the average for humans as 7.5 mg.%; and the range of results for humans as 4.2 to 15.0 mg.% whole blood. In a further paper (5), they state that for some unknown reason, the use of tungstomolybdic acid as the deproteinizing agent results in higher ergothioneine levels than when tungstic acid is used.

Rockwood, Turner, and Pfiffner (23), in a paper concerned with substances giving a positive uric acid test, give some data on blood ergothioneine levels. They express their results in terms of uric acid. Since Benedict, et al, (6) state that where the silver salt of ergothioneine is dissolved by cyanide as the base, uric acid produces 7.5 times as much color in the uric acid test as ergothioneine, the results of Rockwood, et al, can be expressed as mg.% ergothioneine by multiplying their

figures by 7.5.

Some of their averages are:

Normal humans - 5.3 mg.% whole blood

Diabetics - 7.5 mg.% whole blood

Dogs - 3.0 - 3.8 mg.% whole blood

Rabbits - 3.0 - 7.5 mg.% whole blood

Renewed interest in ergothioneine was shown in the work of Lawson and Rimington in 1947 (20). Because of the similarity in molecular structure of ergothioneine to thiouracil and its derivatives used clinically. Lawson and Rimington attempted to show that ergothioneine would exhibit an antithyroid effect similar to that of the thiouracil type drugs. Since ergothioneine is a normal constituent of the blood, it was hoped that it would not possess the toxic properties of thiouracil or other similar antithyroid compounds. While the evidence of this work was apparently negative or at any rate doubtful, especially in view of the results published by Astwood and Stanley (1) and Wilson and McGinty (25), renewed interest in this blood constituent had been aroused.

As a result, a rather comprehensive study of ergothioneine was begun in this Department, in the summer of 1948, to attempt to explain the presence of this compound in blood.

b) Scope of Thesis

When the study of ergothioneine was begun, the exact nature of the problems to be investigated were not decided on until some preliminary work could be done to give some indication as to what significant aspects of ergothioneine should be studied. This preliminary work involved a reinvestigation of the Hunter diazo test for the estimation of ergothioneine in simple solutions and blood. In addition, the preparation of some pure ergothioneine was necessary. The details of this research have been published by Hunter (13,18). As a result of this preliminary work, two main problems were established as the basis of this thesis. They were:

1) The determination of the ergothioneine content of ergots from various plant hosts.

Since pure ergothioneine was needed for the investigations, and the yields from blood were insatisfactory (18), some indication of the quantities which might be isolated was deemed advisable.

2) The determination of blood ergothioneine levels in various species, in normal and abnormal conditions.

Following the modification of his original diazo test for ergothioneine (13) by Hunter, and the possible inaccuracy of his previously published data, the compilation of new data concerning the blood levels of

various species, especially of those animals which might be utilized in subsequent experiments, was necessary.

The data on this second phase of the problem, indicated that the blood ergothioneine level in diabetics was higher than the normal. This had also been noted by Benedict et al (5) and also by Rockwood, Turner, and Pfiffner (23). Therefore another problem was added to the thesis:

3) An investigation of the effect of alloxan diabetes on the ergothioneine level in rats.

Because the elevated ergothioneine level in diabetes was persistent over a period of weeks, and because it was shown by Lazarow (21) that certain sulfhydryl compounds protected rats against alloxan-induced diabetes, a trial experiment was attempted to see if ergothioneine exhibited a similar protecting effect.

2. The ergothioneine content of ergot from various plant hosts.

a) Experimental

The method used in preparing filtrates from ergot sclerotia is similar to the initial steps of the process used earlier to isolate ergothioneine from ergot. (14, 15, 17). The ergot is ground and boiled in a dilute acetic acid solution to extract the ergothioneine and to coagulate the protein. Uranium acetate is added to precipitate further protein. Any excess uranium is precipitated by the addition of phosphate ion. A Hunter diazo test is then done on the resulting supernatant.

1) Preparation of test solution

A sample of 30 to 50 mgm. of the ergot is weighed out. If the sclerotia are large, they are divided, if small, as from Calamagrostis canadensis, 20 to 30 sclerotia may be necessary for the sample. The sample is placed in a 15 ml. graduated centrifuge tube, about 4 ml. water and one drop of 2 N acetic acid are added and the tube is then placed in a boiling water bath for 10 minutes. The contents of the tube are then poured into a small mortar and finely ground. This ground mixture is then wholly transferred back

into the original tube using an additional 5 - 6 ml. water. The mixture is replaced in the boiling water bath for a further five minutes and then it is centrifuged. The total volume is noted here for the dilution factor in calculating the ergothioneine content. The supernatant is now carefully transferred to an ordinary centrifuge tube and a slight excess of saturated uranium acetate is added. (The excess is determined by adding a drop of the supernatant to 5% potassium ferrocyanide on a test plate.) From 0.1 - 0.2 ml. of uranium acetate is usally necessary. The tube is again centrifuged, the supernatant is decanted into another similar tube and the slight excess of uranium precipitated by the addition of one drop (0.05 ml.) of 10% NaH_2PO_4. The solutions are mixed and allowed to stand 15-20 minutes for complete precipitation before centrifuging. The supernatant is decanted into another tube for testing.

2) Colorimetric determination

The ergothioneine content of aliquots of the test filtrates prepared as above, is determined by means of the modified Hunter diazo test for ergothioneine, the details of which are presented in section 3. of this thesis.

b) Results - (see Table I)

Table I. (17)

The ergothioneine content of air-dried sclerotia classified according to host plant.

Host	%Ergothioneine	
	In separate samples	Average
Agropyron Smithii Rydb.	0.180 0.261	0.220
Agropyron repens (L.) Beauv.	0.333 0.430 0.305	0.356
Bromus inermis Leyss	0.381 0.362 0.530	0.424
Calamagrostis canadensis (Michx.) Beauv.	0.162 0.157	0.160
Elymus innovatus Beal	0.310 0.329	0.320
Hordeum vulgare var. trifurcatum Schlecht	0.334 0.318 0.385	0.344
Hordeum vulgare L. (immature 1948)	0.483 0.531 0.442	0.485
Hordeum vulgare L. (mature 1948)	0.325 0.455 0.372	0.384

cont.

Table I. (continued)

Host	% Ergothioneine	
	In separate samples	Average
Hordeum vulgare L. (mature 1947)	0.231 0.298	0.265
Secale cereale L. 1948- Drumheller	0.299 0.183 0.215	0.232
Secale cereale L. 1948- Edmonton (mature)	0.383 0.326 0.368	0.359
Secale cereale L. - 1948- Edmonton (immature)	0.233 0.293 0.202	0.243
Secale cereale L. - 1947- Edmonton (mature)	0.448 0.406 0.401	0.418
Sample T-1 (unknown host)	0.364 0.474 0.445	0.428

c) Discussion

It was found that aliquots of test solution greater than 1 ml. in volume exhibited gross interference, i.e. as the aliquots increased in volume greater than 1 ml., the resulting color and values were less than those given by smaller aliquots. In aliquots smaller than 1 ml. the interfering substance (s) were diluted beyond their range of interference and close proportionality was found between aliquots.

The dilution calculation (see above) for computing the ergothioneine content was not exact. When the material was finely ground, reheated and centrifuged, it was assumed that the ergothioneine was evenly distributed between the solid and liquid. In addition, small amounts of ergothioneine were lost in each subsequent precipitation, while the addition of the uranium and phosphate solutions diluted the filtrate from 1 to 4%. Therefore the values given are probably slightly low.

Except for a few samples, triplicate analyses on single sclerotia were done. There was a wide variability among samples of ergot from the same species of host. The ergots of rye, barley, Bromus and Agropyron contain about the same concentrations of ergothioneine.

The lowest value was found in a sample of ergot from Calamagrostis but here the sclerotia were minute (1 mgm. each) and some foreign materials may have been present to lower the value.

3. The blood ergothioneine levels of various species.

a) Experimental

1) The preparation of test solution.

a. Reagents

Acetic acid - oxalate solutions

For the deproteinization of whole blood, corpuscles, and plasma in 1:10 and 1:5 dilutions.

1:10 dilution

Whole blood	1 vol. plus 9 vols.	0.0045 N acetic acid
Corpuscles	1 vol. plus 9 vols.	0.0030 N acetic acid
Plasma	1 vol. plus 9 vols.	0.0055 N acetic acid

To the above solutions are added 160 mgm. sodium oxalate per liter.

1:5 dilution

Whole blood	1 vol. plus 4 vols.	0.0100 N acetic acid
Corpuscles	1 vol. plus 4 vols.	0.0070 N acetic acid
Plasma	1 vol. plus 4 vols.	0.0125 N acetic acid

To the above solutions were added 360 mgm. sodium oxalate per liter of solution. Ordinarily the reagents for the determination of ergothioneine in plasma were not required since only the cells contain ergothioneine.

Goulard's Extract (11)

220 gms lead acetate -- $Pb(CH_3COO)_2.3H_2O$

140 gms lead monoxide -- litharge (PbO)

Approximately 1 liter of CO_2 free distilled water

Boil 30 minutes with stirring or shaking. Cool and filter (gravity) into a one liter volumetric flask. Wash the filter paper and contents with CO_2 free water and make up to volume. Keep the solution well stoppered.

Phosphate solution

An aqueous 10% solution of dihydrogen sodium phosphate ($NaH_2PO_4.H_2O$)

b) Deproteinization of blood

The preparation of protein-free filtrates by means of the commonly used and suggested alkaloidal agents was shown by Hunter (13) to be unsatisfactory. Filtrates prepared by these reagents often show interference with the diazo test for ergothioneine, and even when interference is absent, the results fluctuate although the same blood is used. For these reasons a new deproteinizing method was devised (13). This simple technique involves heating the blood with a weak acetic acid solution. The strength of acid used for whole blood is a compromise between the

concentrations necessary to bring the serum proteins and the hemoglobin to their respective isoelectric points. Despite such rather apparent shortcomings, the method in practice was found to be quite satisfactory for whole blood, plasma, and cells.

c. Lead acetate precipitation

The basic lead acetate preparation known as Goulard's Extract (11) was used to precipitate reduced glutathione. Glutathione causes serious interference with the subsequent diazo test, and must be removed from the filtrate. It was found that proper precipitation of glutathione with the Goulard's was promoted by the presence of oxalate ion in small concentrations. Ordinarily the excess oxalate present in the blood samples as collected was sufficient, but to ensure uniform precipitation, oxalate was incorporated into the standard acetic acid solutions used for deproteinization. (see above)

d. Method (whole blood, 1:10 dilution)

To 0.5 ml. well mixed whole blood in a 16 ml. centrifuge tube add 4.5 ml. of the acetic acid - oxalate mixture and mix with a stirring rod. Place in a gently boiling water bath and stir. Coagulation takes place in 1-2 minutes. By thoroughly stirring and rubbing the walls of the tube, the coagulated

protein is loosened and gas bubbles released, ensuring more thorough packing in the ensuing centrifugation. Following centrifugation the supernatant is decanted into another similar tube.

To the supernatant add one drop (0.05 ml.) of Goulard's extract. Ensure thorough mixing by rotating and tilting the tube to bring isolated droplets into contact with the main body of fluid. Centrifuge. Decant into a third centrifuge tube. Add one drop of phosphate solution, mixing thoroughly as before. Centrifuge. Decant into a test tube. This is the completed test solution ready for the estimation of ergothioneine.

2) Colorimetric Determination of Ergothioneine.

a. Reagents

Diazo reagent (according to Koessler and Hanke (19)

Into a 50 ml. volumetric flask immersed in ice water add: 1.5 ml. of a solution containing 9 gm. sulfanilic acid and 90 ml. 37% HCl per liter.

1.5 ml. 5% sodium nitrite, and mix.

After 5 minutes add a further 6 ml. 5% $NaNO_2$.

After another 5 minutes ice-cold water is added to the mark, and the contents are thoroughly mixed by

repeated inversion. The solution is ready for use after fifteen minutes.

Alkaline buffer

1 gm. anhydrous sodium carbonate
10 gm. anhydrous sodium acetate
in 100 ml. aqueous solution.

Ten Normal sodium hydroxide

Prepared from saturated, technical grade NaOH which has been allowed to stand two or more weeks until carbonates impurities, etc. settle out. The technical grade NaOH was found to be satisfactory.

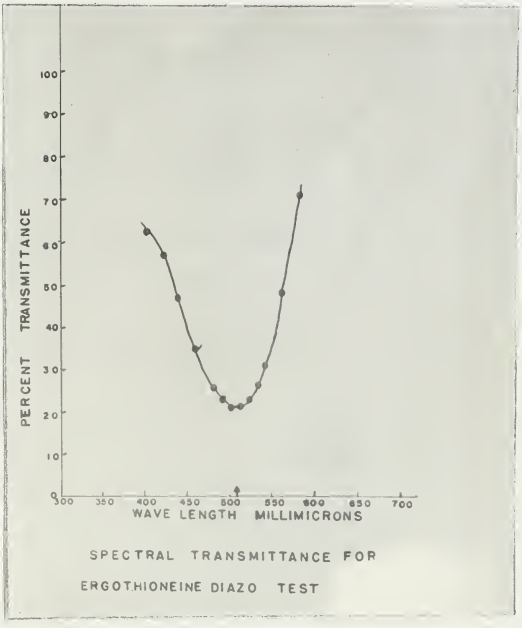
b. Method

If an X ml. aliquot of the test solution is to be used, (2 - X) ml. of water is placed in a calibrated colorimeter tube. Place the tube in the ice-water surrounding the diazo reagent. Then add to the tube: 1 ml. diazo reagent, then 2 ml. of the alkaline buffer solution.

As rapidly as possible add X ml. of the test solution. Thoroughly mix the contents of the tube by tilting and rotating. When ergothioneine is present a clear lemon-yellow color develops. 45 seconds after the addition of the test solution the tube is removed from the ice-water and 5 ml. of 10 N NaOH is

Figure 1 .

Spectral absorption curve of the color produced in the ergothioneine diazo test.



rapidly added and thoroughly mixed by vigorous shaking. The tube is set aside for three to five minutes at which time the solution is free of bubbles and the purplish-red color has fully developed. A reading is now taken in an Evelyn photoelectric colorimeter, with filter 520, using a blank, consisting of a test carried out on 2 ml. of water, to set the galvenometer at 100% transmittance. This blank is stable for at least a week if the tube is kept stoppered. (Check for etching of the glass by the strong base of the test)

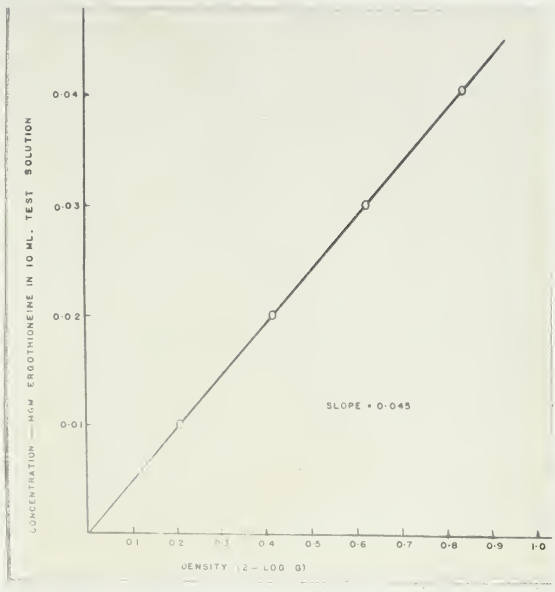
The color of the test solutions shows little fading over a period of 30 minutes.

Calibration Curve

A spectral absorption curve (see figure 1) of the color produced in the diazo test for ergothioneine was determined by means of a Coleman spectrophotometer, Model 11. This curve shows a maximum absorption at close to 510 mμ. Therefore an Evelyn filter #520, transmitting in the range 495 to 550 mμ was chosen as sufficiently selective for the determination.

A calibration curve (see figure 2) was drawn up using various dilutions and aliquots of a standard, pure ergothioneine solution. If the concentration of

Figure 2



Calibration Curve for Ergothioneine Determination

to the colorimetric density measured as ergothioneine in whole blood. This error due to the plasma was found to be remarkably constant in human blood, (the only species for which the effect was accurately determined). This correction was therefore applied to the human values. The values for other species were not corrected, and therefore the levels given

ergothioneine in the 10 ml. test solution is plotted against the "density" (2 - log G) where G is the logarithm (base 10) of the galvanometer reading in per cent, a straight line results, the slope of which is 0.045. In practice, a table was made up in which the mg. % ergothioneine in a 1:10 dilution of the original material was read directly from the galvanometer reading.

Blood filtrates prepared as described above were found to give in almost all cases (notable exceptions are discussed later) true proportionality for different aliquots of filtrate. In addition ergothioneine added to such bloods was quantitatively determined in the filtrates. (13)

The cell volume of all blood samples was determined by centrifuging the blood in Wintrobe hematocrit tubes at 3000 r.p.m. for 25 - 30 minutes. The result obtained was used to express the ergothioneine values in terms of 100 ml. of corpuscles since the ergothioneine is present only in the corpuscles. (17)

Subsequent to the determination of many of the whole blood ergothioneine levels in this project, other researchers in this laboratory found that plasma, which produces a yellow color with the diazo test, contributes

are presumably high.

The color produced by human plasma was found to be equivalent to 1.0 ± 0.2 mg.% ergothioneine. A fraction of an average value of 1.0 mg. %, proportional to the plasma volume in per cent, was subtracted from each respective value for human whole blood.

b) Results. (see Table II)

Table II

The ergothioneine level of various species.

(Expressed in mgm./ 100 ml. corpuscles.)

Species	No. of det.	Range	Mean	St.Dev.	St.Error
Rat -male normal	38	5.2 - 16.5	11.2	2.9	0.48
Rat -male diabetic	18	11.7 - 26.0	21.1	4.6	1.12
Rat -female normal	8	6.6 - 17.6	14.3	6.2	
Hogs	10	11.9 - 18.6	15.7	2.0	
Rabbit	10	7.9 - 21.3	15.1	4.0	
Horse	10	25.3 - 51.0	38.2	6.1	
Human (corrected for plasma error)	94	3.9 - 17.7	9.6	3.1	0.32

c) Discussion

The data for the male rats in Table II show that the diabetic animals have a higher level than the normal group. These diabetics were animals being discarded from other experiments and had been diabetic at least eight weeks. It was because of this evidence that the experiments in sections 4 a) and 4 b) of this thesis were undertaken since the diets and conditions had varied from the normal and no controls could be obtained. The small group of normal female rats showed a wide "scatter" in the distribution of their results, so that the data are not likely to be significant.

The human values were all corrected for the plasma error as explained in this section under method. These bloods were collected from university students and Red Cross blood donors.

Determinations were attempted on several other species. Sheep and cattle bloods showed gross interference with the diazo test. The coupling color was yellow, but on the addition of the strong base, very little intensification of color resulted, the final color being a dull bronze shade. Combined uric acid, as shown by Bulmer, Eagles and Hunter (8) and Davis,

Newton and Benedict (9) to be present in ox blood may possibly be the cause of the interference. Mr. A Harper of this department last summer isolated a substance from ox blood which in small amounts would cause interference similar to that shown in ox and sheep blood when added to filtrates in which no interference had previously been found. This unknown substance appeared to be a nucleoside of uric acid.

Several avian bloods were tested. In about 60% of the specimens interference of varying degree occured, from slight discrepancies between aliquot determinations, to complete inhibition of change of the yellow coupling color. In many cases the coupling color was atypical. Mr. K. A. Siluch showed that ergothioneine was present in these filtrates by means of paper chromatograms.

The blood of two frogs was tested. The diazo test was unsuccessful. The colors produced were similar to those given by histidine hydrochloride or histamine, i.e. a deep orange coupling color and an orange-red when the strong sodium hydroxide is added.

Several attempts were made to obtain a positive ergothioneine test from filtrates prepared by various means from two specimens of human mammary cancer tissue, with no success. Whether this was due to the absence of ergothioneine or faulty preparations of the filtrates is not known.

4a. The Effect of Alloxan Diabetes on the Ergothioneine Level in Blood of Rats.

a) Experimental

The ergothioneine values of a group of normal, mature, male albino rats were determined. Part of the group were then injected with alloxan to produce diabetes. (A 3.2% solution of alloxan monohydrate was used, given in a single subcutaneous injection of 160 mg./Kg body weight.) After five days the blood sugar levels were determined by Reinecke's method (22) to show whether the animals were diabetic or not. The rats were allowed food and water ad lib. The food consisted of a finely ground mixture of equal parts of Purina fox checkers and Victor fox cubes.

In the first test group, ergothioneine determinations were done every week following the injection. It was found that little change in the ergothioneine level occurred until the third week following the injection. Therefore in a subsequent group the determinations were done at two week intervals for six weeks. At the end of each experiment the blood sugars were again determined to indicate whether the diabetes still persisted. The results for the two groups of alloxan diabetic and control animals are combined and presented in Table III.

b) Results

TABLE III

A comparison of the blood ergothioneine levels of alloxan-diabetic and normal rats.

All results are expressed in mgm. ergothioneine per 100 ml. corpuscles

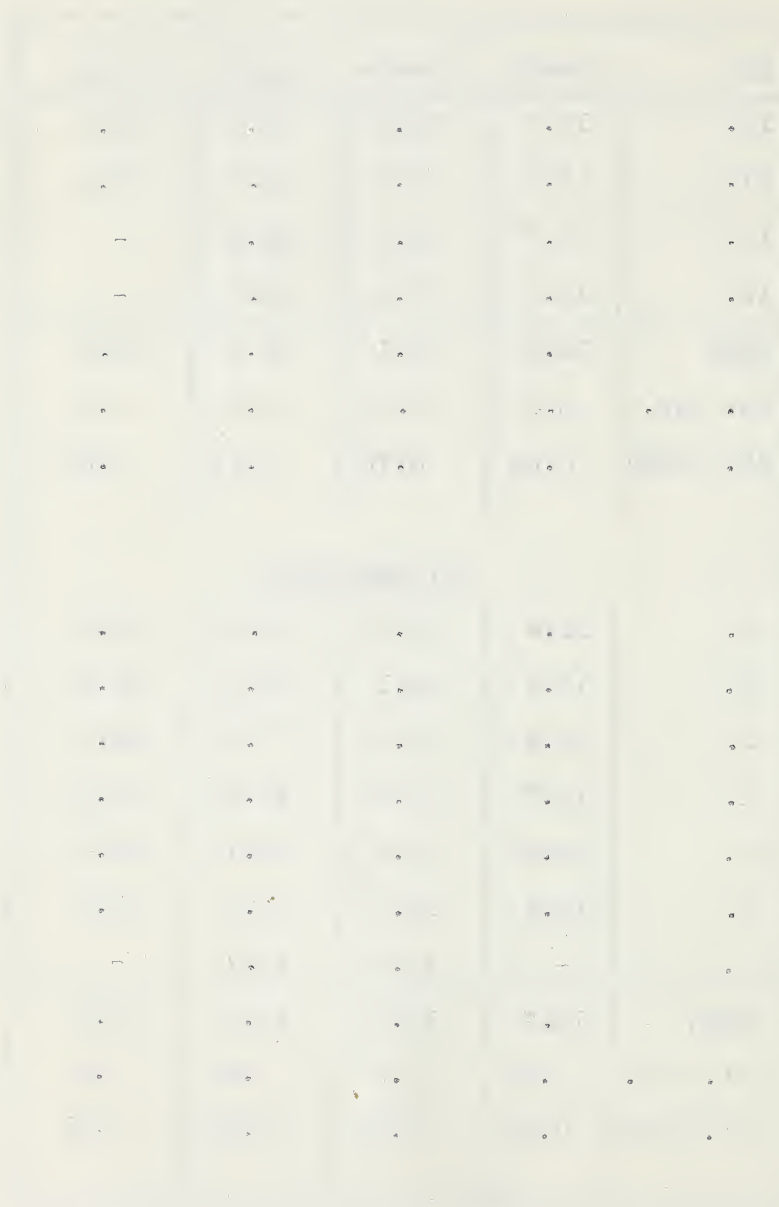
a) Diabetics

Rat	0 weeks	2 weeks	4 weeks	6 weeks
1.	14.6	13.5	20.8	28.0
2.	13.1	13.1	29.6	39.2
3.	12.3	14.7	22.7	-
4.	14.0	11.7	22.4	27.4
5.	14.2	19.3	20.8	19.7
6.	12.1	17.1	20.4	22.4
7.	12.1	13.8	14.4	17.8
8.	14.3	18.2	19.3	24.2
9.	18.4	19.3	20.0	26.3
10.	16.6	22.1	21.2	24.0
11.	13.5	20.8	23.9	30.2
12.	13.9	18.8	16.7	20.4
13.	12.8	18.6	29.0	27.9
14.	9.3	12.7	14.8	16.5
15.	10.4	13.8	17.9	-

(continued next page)

TABLE III (continued)

Rat	0 weeks	2 weeks	4 weeks	6 weeks
16.	10.6	14.8	19.6	20.6
17.	11.3	15.5	24.8	30.2
18.	9.7	10.7	16.2	-
19.	11.9	17.2	22.3	-
Mean	12.9	16.1	20.9	25.0
St. Dev.	2.3	3.2	4.1	5.9
St. Error	0.54	0.75	0.97	1.52
		b) Controls		
1.	12.6	13.6	14.8	17.3
2.	11.8	13.1	15.5	20.0
3.	12.5	16.6	17.0	20.0
4.	11.7	11.5	15.4	16.6
5.	15.0	14.6	18.7	20.4
6.	12.4	13.3	16.3	18.5
7.	-	14.0	15.7	-
Mean	12.7	13.8	16.2	18.8
St. Dev.	1.2	1.5	1.3	1.6
St. Error	0.54	0.61	0.53	0.71



c) Discussion

The means of the two groups indicate that over a period of six weeks the rise in the levels of the diabetic group was almost twice the rise of the control group. This rise of values in the controls and presumably a corresponding rise in the diabetic group could be partially explained by the change in the diet of the experimental animals from the stock mixture fed routinely in the lab, which was predominantly the Victor Fox Cubes. It was found in other projects that the ergothioneine level was raised by increasing the proportion of Purina Checkers in the diet. The data show however, that the diabetic levels increased on the average 50% more than the increase due to dietary effects.

The t values were calculated to determine if the controls and diabetics differed significantly. For example the t value at the 4 week interval was 2.947. The corresponding p value was less than 1%, indicating that there was a highly significant difference between the two groups.

Determinations on several of the diabetics and controls were done beyond the six week period. The diabetic values continued to show a gradual rise,

while the control group average was slowly falling at the eight and ten week intervals.

The reason for this rise of blood ergothioneine in diabetic rats is not known. It has been shown by Dr. R. Fraser of this department that the mean value for human diabetics is significantly higher than the normal. The rise in diabetic rats was thus probably due to the diabetes. This rise may be a result of the general metabolic upset associated with diabetes or perhaps is due to some compensatory mechanism established by the animal. Recent work on the effect of various sulfhydryl compounds on several types of experimentally produced diabetes lends some support to this latter idea. If these sulfhydryl compounds maintain the integrity of essential sulfhydryl groups or disulfide linkages of insulin or of enzymes essential to the formation or function of the secretion of the islets of Langerhans, perhaps the increased ergothioneine concentration in the blood of diabetics is a reflection of an attempt by the body to supply sulfhydryl groups for this purpose. (See section 4b. of this thesis for a more complete discussion and references.)

4b. The Effect of Intravenously-Injected Ergothioneine on Alloxan-Induced Diabetes in Rats.

a) Introduction

Various workers believe that the action of alloxan on the beta cells of the islets of Langerhens is due to the combination of the alloxan with the sulfhydryl groups of proteins, with resulting enzyme inactivation. It is known that alloxan inhibits the enzymes, succinic dehydrogenase, papain, and cathepsin. Active sulfhydryl groups are essential for pyruvate oxidation and condensation, malate and ketoglutarate oxidation, D-amino acid and L-glutamic acid oxidation, and various fatty acid oxidations. (Barron & Singer, J. Biol. Chem. 157:221 & 241:1945).

Many of the enzymes inactivated by alloxan can be reactivated, partially at least, by the addition of glutathione. Therefore Lazarow (21) decided to test the effect of glutathione on the course of alloxan diabetes. Cysteine was also used since it is cheaper than glutathione and because the sulfhydryl group of reduced glutathione is contained in the cysteine portion of its molecule. Lazarow found both cysteine and glutathione in certain concentrations prevented the development of diabetes when injected intravenously,

immediately prior to intravenous injection of alloxan (a 2% solution at a dosage of 40 mg./Kg. body weight). When injected alone, this dosage of alloxan produced a 95% incidence of diabetes. The dosages of cysteine and glutathione found effective in preventing alloxan diabetes were 7.5 millimols per Kg. body weight. This is equivalent to 912 mg. cysteine-/Kg. body weight, and 2500 mg. glutathione/Kg. body weight. (These amounts seem quite large when compared to the present findings with ergothioneine).

Since ergothioneine, like glutathione and cysteine, occurs naturally in the body, and in addition is a sulfhydryl compound, its effect on the development of alloxan diabetes in the rat was studied in a manner analagous to Lazarow's work. However due to the difficulty in obtaining pure ergothioneine in sufficient quantities, the amounts injected were considerably smaller in comparison to those used by Lazarow.

b) Experimental

Eight healthy, male, Wistar strain albino rats of good size (250-300 gms.) were selected. The buttocks and proximal portion of the tail were closely shaven. The initial blood sugars were than determined by Reinecke's method (22) and the rats were left with food and water ad libitum for 24 hours. The next day the following procedure was used to inject the alloxan and ergothioneine intravenously: the tail and rump of the rat were immersed into a beaker of warm water (45 degrees C.) and held there while ether anaesthesia was administered. When unconscious, the rat was removed from the bath, dried, and a tourniquet (an elastic band) applied to the base of the tail. The injection(s) were then made into the engorged tail veins using fine hypodermic needles (5/8" 25 gauge). Blood was drawn into the syringe before the injection was made, to ensure that the vein had been entered. When the vein had been entered the tourniquet was removed and the solution injected.

The rats were divided into three groups and injected as follows:

Group 1 - 4 rats

a) 5 mg. ergothioneine in 0.5 ml. phosphate buffer-pH 7.4.

b) followed in 3-4 minutes by an injection of 2% alloxan monohydrate at a dosage of 40 mg./Kg. body weight.

Group 2 - 2 rats

a) ergothioneine as in Group 1 a)

Group 3 - 2 rats

a) alloxan as in Group 1 b)

Following the injections, the rats were returned to their cage and food and water was plentifully supplied. The blood sugars were determined at 18 and 72 hours after injection.

c) Results

TABLE IV

Effect on Blood Sugar of intravenously injected ergothioneine and alloxan

Rat	Injection	Blood sugar determination		
		24 hrs before injection	18 hrs after injection	72 hrs after injection
	Group 1			
4.	ergothioneine & alloxan	103mg. %	130mg. %	180mg. %
6.	ergothioneine & alloxan	95	275	314 plus
8.	ergothioneine & alloxan	128	120	159
11.	ergothioneine & alloxan	108	149	194
	Group 2			
1.	ergothioneine	105	115	149
2.	"	119	151	-
	Group 3			
3.	alloxan	112	245	295
9.	"	105	314 plus	314 plus

d) Discussion

The thesis of P. Wight indicates that the normal range of rat blood sugars using Reinecke's method is 80 - 200 mg. % glucose. Blood sugars of over 200 mg. % are considered as indicative of mild diabetes, whereas values over 300 mg. % indicate severe diabetes.

One of the four rats injected with both ergothioneine and alloxan showed the hyperglycemia which developed when alloxan alone was injected. The time interval between the injection of the two solutions in the case of rat #6, was about 4½ minutes, while in the case of the other three rats receiving the double injection, the time interval was under three minutes, the interval suggested by Lazarow as being the maximum allowable for cysteine and glutathione to be effective in preventing hyperglycemia. It would appear from this small test that intravenously injected ergothioneine even in such small concentration as 0.087 millimols per Kg body weight, may in some way inhibit the elevation of blood sugar, induced by alloxan.

The blood ergothioneine values were determined at the same time as the blood sugars. No significant change in blood ergothioneine could be found in any of

the various test animals. The small fluctuations observed were no larger than the normal variation seen in daily blood ergothioneine determinations.

5. Summary

1. The ergothioneine content of the ergot sclerotia of various species was determined. These values may be of use in indicating the best sources of ergothioneine from ergots in general.

2. The blood ergothioneine values of groups of several mammalian species are given. It is hoped that these data might be of use if further studies of blood ergothioneine, clinically or experimentally were undertaken.

3. It has been shown that alloxan diabetes in the rat elevates the blood ergothioneine within a month.

4. A test experiment indicated that the intravenous injection of ergothioneine solution at a dosage of 0.09 millimols/Kg. body weight, may protect rats against alloxan-induced diabetes.

5. The possible function or cause of blood ergothioneine still remains to be elucidated. However it is hoped that the data presented in this thesis may be of assistance in solving this problem.

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